

WHAT IS CLAIMED IS:

- 1 1. A four helical bundle (4HB) polypeptide comprising one or more non-naturally encoded amino acids.
- 1 2. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or more post-translational modifications.
- 1 3. The 4HB polypeptide of claim 1, wherein the polypeptide is linked to a linker, polymer, or biologically active molecule.
- 1 4. The 4HB polypeptide of claim 3, wherein the polypeptide is linked to a water soluble polymer.
- 1 5. The 4HB polypeptide of claim 1, wherein the polypeptide is linked to a bifunctional polymer, bifunctional linker, or at least one additional 4HB polypeptide.
- 1 6. The 4HB polypeptide of claim 5, wherein the bifunctional linker or bifunctional polymer is linked to a second polypeptide.
- 1 7. The 4HB polypeptide of claim 6, wherein the second polypeptide is a 4HB polypeptide.
- 1 8. The 4HB polypeptide of claim 4, wherein the water soluble polymer comprises a poly(ethylene glycol) moiety.
- 1 9. The 4HB polypeptide of claim 4, wherein said water soluble polymer is linked to a non-naturally encoded amino acid present in said 4HB polypeptide.
- 1 10. The 4HB polypeptide of claim 1, selected from the group consisting of G-CSF, erythropoietin, interferon, and growth hormone.
- 3 11. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or more amino acid substitution, addition or deletion that modulates affinity of the 4HB polypeptide for a 4HB receptor.

1 12. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or
2 more amino acid substitution, addition or deletion that increases the stability or solubility of the
3 4HB polypeptide.

1 13. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or
2 more amino acid substitution, addition or deletion that increases the expression of the 4HB
3 polypeptide in a recombinant host cell or synthesized in vitro.

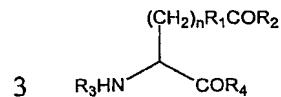
1 14. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or
2 more amino acid substitution, addition or deletion that increases protease resistance of the 4HB
3 polypeptide.

1 15. The 4HB polypeptide of claim 1, wherein the non-naturally encoded amino acid
2 is reactive toward a linker, polymer, or biologically active molecule that is otherwise unreactive
3 toward any of the 20 common amino acids in the polypeptide.

1 16. The 4HB polypeptide of claim 1, wherein the non-naturally encoded amino acid
2 comprises a carbonyl group, an amooxy group, a hydrazine group, a hydrazide group, a
3 semicarbazide group, an azide group, or an alkyne group.

1 17. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 comprises a carbonyl group.

1 18. The 4HB polypeptide of claim 17, wherein the non-naturally encoded amino acid
2 has the structure:



3
4 wherein n is 0-10; R₁ is an alkyl, aryl, substituted alkyl, or substituted aryl; R₂ is H, an alkyl,
5 aryl, substituted alkyl, and substituted aryl; and R₃ is H, an amino acid, a polypeptide, or an
6 amino terminus modification group, and R₄ is H, an amino acid, a polypeptide, or a carboxy
7 terminus modification group.

1 19. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 comprises an amooxy group.

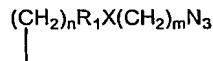
1 20. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 comprises a hydrazide group.

1 21. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 comprises a hydrazine group.

1 22. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 residue comprises a semicarbazide group.

1 23. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 residue comprises an azide group.

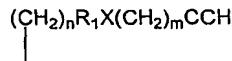
1 24. The 4HB polypeptide of claim 23, wherein the non-naturally encoded amino acid
2 has the structure:



3 wherein n is 0-10; R₁ is an alkyl, aryl, substituted alkyl, substituted aryl or not present; X is O,
4 N, S or not present; m is 0-10; R₂ is H, an amino acid, a polypeptide, or an amino terminus
5 modification group, and R₃ is H, an amino acid, a polypeptide, or a carboxy terminus
6 modification group.

1 25. The 4HB polypeptide of claim 16, wherein the non-naturally encoded amino acid
2 comprises an alkyne group.

1 26. The 4HB polypeptide of claim 25, wherein the non-naturally encoded amino acid
2 has the structure:



3 wherein n is 0-10; R₁ is an alkyl, aryl, substituted alkyl, or substituted aryl; X is O, N, S or not
4 present; m is 0-10, R₂ is H, an amino acid, a polypeptide, or an amino terminus modification
5 group, and R₃ is H, an amino acid, a polypeptide, or a carboxy terminus modification group.

1 27. The 4HB polypeptide of claim 4, wherein the water soluble polymer has a
2 molecular weight of between about 0.1 kDa and about 100 kDa.

- 1 28. The 4HB polypeptide of claim 27, wherein the water soluble polymer has a
2 molecular weight of between about 0.1 kDa and about 50 kDa.
- 1 29. The 4HB polypeptide of claim 4, which is made by reacting a 4HB polypeptide
2 comprising a carbonyl-containing amino acid with a water soluble polymer comprising an
3 amiooxy, hydrazine, hydrazide or semicarbazide group.
- 1 30. The 4HB polypeptide of claim 29, wherein the amiooxy, hydrazine, hydrazide
2 or semicarbazide group is linked to the water soluble polymer through an amide linkage.
- 1 31. The 4HB polypeptide of claim 4, which is made by reacting a water soluble
2 polymer comprising a carbonyl group with a polypeptide comprising a non-naturally encoded
3 amino acid that comprises an amiooxy, a hydrazine, a hydrazide or a semicarbazide group.
- 1 32. The 4HB polypeptide of claim 4, which is made by reacting a 4HB polypeptide
2 comprising an alkyne-containing amino acid with a water soluble polymer comprising an azide
3 moiety.
- 1 33. The 4HB polypeptide of claim 4, which is made by reacting a 4HB polypeptide
2 comprising an azide-containing amino acid with a water soluble polymer comprising an alkyne
3 moiety.
- 1 34. The 4HB polypeptide of claim 16, wherein the azide or alkyne group is linked to
2 a water soluble polymer through an amide linkage.
- 1 35. The 4HB polypeptide of claim 4, wherein the water soluble polymer is a
2 branched or multiarmed polymer.
- 1 36. The 4HB polypeptide of claim 35, wherein each branch of the branched polymer
2 has a molecular weight of between about 1 kDa and about 100 kDa.
- 1 37. The 4HB polypeptide of claim 1, wherein the polypeptide is a 4HB antagonist.
- 1 38. The 4HB polypeptide of claim 37, wherein the polypeptide comprises one or
2 more post-translational modification, linker, polymer, or biologically active molecule.

1 39. The 4HB polypeptide of claim 38, wherein the polymer comprises a moiety
2 selected from a group consisting of a water soluble polymer and poly(ethylene glycol).

1 40. The 4HB polypeptide according to claim 37, wherein the non-naturally encoded
2 amino acid is present within the Site II region of the 4HB polypeptide.

1 41. The 4HB polypeptide according to claim 37, wherein the polypeptide prevents
2 dimerization of a 4HB receptor.

1 42. The 4HB polypeptide of claim 1, wherein the non-naturally encoded amino acid
2 comprises a saccharide moiety.

1 43. The 4HB polypeptide of claim 3, wherein the linker, polymer, or biologically
2 active molecule is linked to the polypeptide via a saccharide moiety.

1 44. An isolated nucleic acid comprising a polynucleotide that encodes a 4HB
2 polypeptide, wherein the polynucleotide comprises at least one selector codon.

1 45. The isolated nucleic acid of claim 44, wherein the selector codon is selected from
2 the group consisting of an amber codon, ochre codon, opal codon, a unique codon, a rare codon,
3 and a four-base codon.

1 46. A method of making the 4HB polypeptide of claim 3, the method comprising
2 contacting an isolated 4HB polypeptide comprising a non-naturally encoded amino acid with a
3 linker, polymer, or biologically active molecule comprising a moiety that reacts with the non-
4 naturally encoded amino acid.

1 47. The method of claim 46, wherein the polymer comprises a moiety selected from a
2 group consisting of a water soluble polymer and a poly(ethylene glycol).

1 48. The method of claim 46, wherein the non-naturally encoded amino acid
2 comprises a carbonyl group, an aminoxy group, a hydrazide group, a hydrazine group, a
3 semicarbazide group, an azide group, or an alkyne group.

1 49. The method of claim 46, wherein the non-naturally encoded amino acid
2 comprises a carbonyl moiety and the linker, polymer, or biologically active molecule comprises
3 an aminoxy, a hydrazine, a hydrazide or a semicarbazide moiety.

1 50. The method of claim 49, wherein the aminoxy, hydrazine, hydrazide or
2 semicarbazide moiety is linked to the linker, polymer, or biologically active molecule through an
3 amide linkage.

1 51. The method of claim 46, wherein the non-naturally encoded amino acid residue
2 comprises an alkyne moiety and the linker, polymer, or biologically active molecule comprises an
3 azide moiety.

1 52. The method of claim 46, wherein the non-naturally encoded amino acid residue
2 comprises an azide moiety and the linker, polymer, or biologically active molecule comprises an
3 alkyne moiety.

1 53. The method of claim 48, wherein the azide or alkyne moiety is linked to a linker,
2 polymer, or biologically active molecule through an amide linkage.

1 54. The method of claim 47, wherein the poly(ethylene glycol) moiety has an average
2 molecular weight of between about 0.1 kDa and about 100 kDa.

1 55. The method of claim 47, wherein the poly(ethylene glycol) moiety is a branched
2 or multiarmed polymer.

1 56. A composition comprising the 4HB polypeptide of claim 1 and a
2 pharmaceutically acceptable carrier.

1 57. The composition of claim 56, wherein the non-naturally encoded amino acid is
2 linked to a water soluble polymer.

1 58. A method of treating a patient having a disorder modulated by 4HB comprising
2 administering to the patient a therapeutically-effective amount of the composition of claim 56.

1 59. A cell comprising the nucleic acid of claim 44.

1 60. The cell of claim 59, wherein the cell comprises an orthogonal tRNA synthetase
2 or an orthogonal tRNA.

1 61. A method of making a 4HB polypeptide comprising a non-naturally encoded
2 amino acid, the method comprising, culturing cells comprising a polynucleotide or
3 polynucleotides encoding a 4HB polypeptide and comprising a selector codon, an orthogonal
4 RNA synthetase and an orthogonal tRNA under conditions to permit expression of the 4HB
5 polypeptide comprising a non-naturally encoded amino acid; and purifying the 4HB
6 polypeptide.

1 62. A method of increasing serum half-life or circulation time of a 4HB polypeptide,
2 the method comprising substituting one or more non-naturally encoded amino acids for any one
3 or more naturally occurring amino acids in the 4HB polypeptide.

1 63. A 4HB polypeptide encoded by a polynucleotide, wherein said polynucleotide
2 comprises a selector codon, and wherein said polypeptide comprises at least one non-naturally
3 encoded amino acid.

1 64. The 4HB polypeptide of claim 63, wherein the non-naturally encoded amino acid
2 is linked to a linker, polymer, water soluble polymer, or biologically active molecule.

1 65. The 4HB polypeptide of claim 64, wherein the water soluble polymer comprises
2 a poly(ethylene glycol) moiety.

1 66. The 4HB polypeptide of claim 63, wherein the non-naturally encoded amino acid
2 comprises a carbonyl group, an aminoxy group, a hydrazide group, a hydrazine group, a
3 semicarbazide group, an azide group, or an alkyne group.

1 67. The 4HB polypeptide of claim 65, wherein the poly(ethylene glycol) moiety has a
2 molecular weight of between about 0.1 kDa and about 100 kDa.

1 68. The 4HB polypeptide of claim 65, wherein the poly(ethylene glycol) moiety is a
2 branched or multiarmed polymer.

1 69. The 4HB polypeptide of claim 68, wherein the poly(ethylene glycol) moiety has a
2 molecular weight of between about 1 kDa and about 100 kDa.

1 70. A composition comprising the 4HB polypeptide of claim 63 and a
2 pharmaceutically acceptable carrier.

1 71. A 4HB polypeptide comprising one or more amino acid substitution, addition or
2 deletion that increases the expression of the 4HB polypeptide in a recombinant host cell.

1 72. A 4HB polypeptide comprising a water soluble polymer linked by a covalent
2 bond to the 4HB polypeptide at a single amino acid.

1 73. The 4HB polypeptide of claim 72, wherein the water soluble polymer comprises
2 a poly(ethylene glycol) moiety.

1 74. The 4HB polypeptide of claim 72, wherein the amino acid covalently linked to
2 the water soluble polymer is a non-naturally encoded amino acid.

1 75. The 4HB polypeptide of claim 10 wherein said non-naturally encoded amino acid
2 is linked to a poly(ethylene glycol) molecule.

1 76. A polypeptide comprising at least one linker, polymer, or biologically active
2 molecule, wherein said linker, polymer, or biologically active molecule is attached to the
3 polypeptide through a functional group of a non-naturally encoded amino acid ribosomally
4 incorporated into the polypeptide.

1 77. The polypeptide of claim 76, wherein said polypeptide is monoPEGylated.

1 78. The polypeptide of claim 76, wherein said polypeptide is a 4HB polypeptide.

1 79. A polypeptide comprising a linker, polymer, or biologically active molecule that
2 is attached to one or more non-naturally encoded amino acid wherein said non-naturally encoded
3 amino acid is ribosomally incorporated into the polypeptide at pre-selected sites.

1 80. The polypeptide of claim 79, wherein said polypeptide is a 4HB polypeptide.

1 81. The 4 HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or
2 more amino acid substitution, addition, or deletion that modulates immunogenicity of the 4HB
3 polypeptide.

1 82. The 4HB polypeptide of claim 1, wherein the 4HB polypeptide comprises one or
2 more amino acid substitution, addition, or deletion that modulates serum half-life or circulation
3 time of the 4HB polypeptide.

1 83. A method of modulating immunogenicity of a 4HB polypeptide, the method
2 comprising substituting one or more non-naturally encoded amino acids for any one or more
3 naturally occurring amino acids in the 4HB polypeptide.